UNIVERSITY OF MIAMI
ROSENSTIEL SCHOOL OF MARINE AND ATMOSPHERIC SCIENCE

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PHYSALIA TOXIN AND THE ACTIVITY OF BIOLOGICAL MEMBRANES

by

Charles E. Lane

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Dr. John S. Bunt, Chairman
Division of Functional Biology

Dr. F. G. Walton Smith
Dean

ML 70113
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ABSTRACT

The nematocysts of the Portuguese Man-of-War and of the stinging coral, Millepora alcicornis, have been shown to contain a complex protein toxin that acts on ion transport mechanisms in a wide variety of organisms, influencing such functions as conduction in nerve trunks, coordination of vertebrate and invertebrate heart, absorption of amino acids from the gut, ionic and osmotic regulation in marine crustaceans, and sodium transport by surviving frog skin. Some of its chemical and pharmacological properties are described. A locally abundant sponge, Haliclona viridis, provides a potent, non-protein toxin, not yet studied pharmacologically, but lethal to test animals in microgram per kilo quantities. Some of its chemical characteristics are described.

PHYSALIA TOXIN AND THE ACTIVITY OF BIOLOGICAL MEMBRANES

Duration of Project

This research project was initiated in 1967 and was extended to August 31, 1969, and was supported by the Office of Naval Research for the total funding of $25,452.00.

Our studies of Physalia toxin supported by the Office of Naval Research and other agencies extend over several years and have evolved from a simple effort to establish its range of lethality in a variety of animals to an attempt to describe the pharmacology in more quantitative terms. It was early established that this protein material is lethal to all Metazoan animals(1). Interestingly enough, it proved to be an acceptable carbon source for Paramecium and for Tetrahymena(2), but for all higher animals this nematocyst toxin is lethal in low concentration. The LD_{50} for mammals ranges from 50 to 75 μg/kilo for rodents(3) to 75-80 μg/kilo for the dog(4).

In small Crustacea such as the fiddler crab, Uca pugilator, the injection of 5 to 7 μg of Physalia toxin into the hemocoele results in paralysis and death within minutes. In a larger specimen, such as the land crab, Cardisoma guanhumi, in which changes in the electrocardiogram and in hemocoelic pressure may be readily monitored, it was noted that the first effect the toxin evoked was cardiac arrest(5). With proper placement of electrocardiographic electrodes the potentials generated by each of the few cells in the intrinsic cardiac ganglion could be recorded. When the animal was injected with Physalia toxin the output of action potentials by the cardiac ganglion cells was

augmented but the heart did not respond to these signals. In such a preparation, if the heart were led by external stimulation it contracted normally. This suggested that the heart, although competent to contract, was prevented from doing so by some interruption in the normal conduction pathway between the cell bodies of the cardiac ganglion and the heart muscle.

In the isolated frog sciatic-gastrocnemius preparation, toxin applied to a limited segment of the sciatic nerve prevents conduction through that segment(6). Electric stimulation of the nerve downstream from the treated segment elicits normal contraction, indicating that another effect of Physalia toxin may be to prevent conduction along nerve axons.

When Physalia toxin is administered intravenously in the rat, a rapid sequence of electrocardiographic changes is initiated. These changes include P-wave modifications, prolongation of the PR-interval, evidence of atrio-ventricular block, the establishment of idioventricular rhythms, ventricular fibrillation, and death. These changes all occur within approximately ten seconds(3). At a dose level of approximately 80 μg/toxin/kilo post-mortem blood collections were extensively hemolyzed. These studies were subsequently extended to include the dog. In this animal the same qualitative sequence of electrocardiographic changes was observed after administration of sublethal doses of toxin. In the dog, hemolysis was a consistent finding, occurring within seconds of intravenous injection of toxin, causing us to investigate changes in the ionic composition of the plasma and in the population of circulating erythrocytes. Plasma potassium levels were elevated by as much as 30% over control values. Plasma sodium values were depressed by a somewhat smaller amount. The numbers of erythrocytes in peripheral blood did not change significantly but the hematocrit was elevated in every experimental dog.

It was early noted that the administration of successive sublethal doses of toxin to the same anesthetized dog produced varying responses. The electrocardiographic changes described were always observed following the initial injection. Subsequent injections of this same dose level were uniformly without effect on the electrocardiogram. Indeed, it was often possible to inject 3 - 5 lethal doses without seriously affecting the electrocardiographic picture.

The failure of the dog to respond to a second injection of *Physalia* toxin appeared to reflect some kind of protection afforded by the initial injection. Since plasma chemistry changed primarily in the direction of increased potassium levels after toxin treatment, we decided to examine the effect of intravenous administration of potassium salts. After an electrocardiographic abnormality had been established and had persisted for 30 minutes, an injection of 10 ml of isotonic K Cl was administered, causing an immediate restoration of the electrocardiogram to normal.

These observations suggest: (1) that *Physalia* toxin affects a wide range of biological material; and (2) that it appears to influence the active ion transport capacity of boundary membranes. Thus, blockade of transmission along the sciatic nerve of the frog could result from interference with sodium conductance, as in tetrodotoxin poisoning[7]. The precise mechanism of *Physalia* toxin action at this site has not yet been established. The toxin-induced changes in the electrocardiogram of various vertebrates can be attributed largely to alterations in the capacity for active transport of sodium and potassium in various components of the pacemaker and conduction systems of the heart. Hemolysis, in our view, results from a toxin-evoked inhibition of ion pumps in the boundary

membrane of the erythrocyte, permitting potassium to diffuse down its electrochemical gradient, upsetting the osmotic balance between the intracellular compartment and the surrounding plasma. Since intracellular sodium in the dog approaches plasma sodium concentration, the sodium distribution was altered somewhat less by toxin. The now hypertonic cell contents absorb osmotic water, leading to an increase in cell volume and ultimately to cell rupture, liberating the hemoglobin into the plasma, and accounting in large measure for the change in sodium and potassium levels in blood plasma after toxin administration. Earlier studies, with a less well-defined toxin preparation, showed no hemolysin activity in vitro[1].

The dramatic curative effects observed after the administration of potassium chloride in the dog are somewhat more difficult to explain, but they reinforce our suggestion that the principal effect of Physalia toxin is exerted on boundary membranes.

Further evidence relating the activity of Physalia toxin to boundary membranes was revealed in a study of ionic regulation in Cardisoma guanhumi. This animal regulates the ionic composition of its internal environment over a wide range of external salinities. Most ion regulation is attributed to the operation of active transport machinery in the gill[8]. This tissue contains an ATPase enzyme complex similar in many respects to that described from brain and erythrocytes of mammals[9].

Physalia toxin inhibits the Na+, K+, and Mg+ stimulated ATPase complex, thereby differing from ouabain that inhibits only the sodium and potassium stimulated components of this enzyme complex.

In addition to studies on the nematocyst toxin of Physalia and other Coelenterates, we have also examined other marine invertebrates with a view to determining their capability of synthesizing products that might be toxic to higher animals. One animal that showed particular promise is the green sponge, *Haliclona viridis*. Our studies confirm that this sponge produces a potent, water soluble toxin that we have extracted and partially purified.

The green sponge is collected in shallow water in the rocky area of Bear Cut (north end of Key Biscayne). Samples are freed of detritus by swirling in sea water, then immediately placed in 3 liter jars of 0.066 M phosphate buffer (Sorensen's), pH 5.8. The buffer quickly becomes dark green and rather viscous. Each sample may be extracted three or four times with fresh buffer. Distinct advantages of this collection and extraction procedure include:

1. Lipids are not extracted, thus markedly facilitating purification of the toxic components.
2. Biological activity is preserved by using a cold rather than a hot extraction technique. The toxic principle is known to be reasonably heat stable, but prolonged exposure to temperatures around 100°C reduces its activity and burdens the purification with unwanted organic materials.
3. Bulky and friable sponge is not transported to the laboratory, but is extracted in the field before chemical deterioration can begin.
4. Natural populations are not decimated and the ecosystem is minimally disturbed since only small sized samples are employed.

The green buffer extracts are clarified by filtration through "Celite" and Whatman #3 paper, dialyzed against several changes of Sorensen's buffer at pH 5.8 in the cold for 24 hours and finally filtered through 1.2 micrometer Millipore membranes. When the clear dark green solution is dialyzed against distilled water and lyophilized, the resulting solids, when injected intraperitoneally into 20 gm Swiss mice, showed an LD_{100} of 500 micrograms per kilo. The lyophilization step causes no significant loss of activity.

Alternatively the solution may be filtered through a column of carboxymethyl cellulose on which toxic activity is retained. The toxin is eluted from this column by 0.2 - 0.3 M sodium chloride in Sorensen's buffer at pH 5.8 at room temperature. Three active peaks may be separated by using 0.2, 0.25, and 0.3 M sodium chloride solutions in succession. TLC separations of each fraction on microcrystalline cellulose developed with n-butanol, acetone, acetic acid, 5% aqueous ammonia, and water (7:5:3:3:2) shows single spots (Iodine vapor) for all fractions. All have the same r_f value (0.74) suggesting that they may in fact represent the same substance, even though they were eluted separately from the column over a narrow range of increasing molarity. This anomalous behavior requires further investigation.

Dialysis of each fraction against distilled water followed by lyophilization produces an amorphous tan solid having no discrete melting point. The LD_{100} of this material is 25 μg/kilo.

The chemical behavior and ultraviolet absorption spectrum of H. viridis toxin suggest that it is not a protein. Partially purified samples of toxin have little absorption at 280 nanometers relative to

260 nanometers. The lethality of an aqueous solution of the toxin is maintained throughout laboratory operations which, in our experience with the protein toxins of <u>Physalia</u>(10) and <u>Miller ora</u>, would destroy it if it were chemically similar. The toxin is stable to moderate heat and pH changes. Although it is insoluble in organic solvents, an aqueous solution may be extracted with organic solvents (petroleum ether, chloroform, and n-butanol) without destruction of activity. Removal of residual solvent from the aqueous phase at 50°C does not affect activity. Although the toxin is non-dialyzable, and thus could have a high molecular weight, similar behavior is exhibited by a non-protein marine toxin, Palytoxin, which is throught to have a molecular weight in the range 1100 - 1500[7].

Toxin obtained from carboxymethyl cellulose still retains some pigment, but it is doubtful that this coloration is part of the pure compound. Similarly, the amorphous character of the toxin and absence of a discrete melting point are disconcerting, and suggest the presence of a single substance from carboxymethyl cellulose.

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Graduate Research Assistants that were salaried under contract:

Dr. James B. Larsen

Dr. Ronald L. Smith

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Larsen, James B. 1968. Some effects of *Physalia physalis* toxin on active transport. Doctoral Dissertation, University of Miami.

Smith, Ronald L. 1967. Protein digestion and the resulting amino acid distribution in the digestive tract of the white grunt, *Haemulon plumieri*. Master's Thesis, University of Miami.

Smith, Ronald L. 1968. Some aspects of digestion and absorption in the white grunt, *Haemulon plumieri* (Pisces: Pomadasyidae). Doctoral Dissertation, University of Miami.

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13. ABSTRACT

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